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Pharmacodynamics of Anidulafungin against Clinical *Aspergillus fumigatus* Isolates in a Nonneutropenic Murine Model of Disseminated Aspergillosis

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Azole resistance is an emerging increasing problem in *Aspergillus fumigatus* that results in treatment failure. Alternative treatments may improve the therapeutic outcome in patients with azole-resistant invasive aspergillosis (IA). Little is known about the *in vivo* efficacy of the echinocandin anidulafungin (AFG) in IA. The *in vivo* efficacy of 2.5, 5, 10, and 20 mg/kg of body weight AFG was assessed against two clinical *Aspergillus fumigatus* isolates with identical AFG minimum effective concentrations (MECs; 0.03 mg/liter) in a murine model of IA: a wild-type voriconazole (VCZ)-susceptible (VCZ^s) *A. fumigatus* isolate (AZN 8196) and a VCZ-resistant (VCZ^r) *A. fumigatus* isolate (V52-35) harboring the TR_{34}/L98H resistance mechanism (substitution at codon L98 in combination with a 34-bp tandem repeat in the promoter region of the *CYP51A* gene). The pharmacokinetics of AFG were also assessed for each dose. Increasing doses increased survival for both isolates in a manner dependent on the AFG dose level ($R^2 = 0.99$ and 0.95, respectively) up to a maximum of 72.7% and 45.45% for the VCZ^s and VCZ^r isolates, respectively. The area under the concentration-time curve (AUC) correlated significantly with the dose in a linear fashion over the entire dosing range ($R^2 = 0.86$). The Hill equation with a variable slope fitted the relationship between the 24-h AUC/MEC ratio and 14-day survival well ($R^2 = 0.87$; $P < 0.05$). The 50% effective AUC/MEC for total AFG was 126.5 (95% confidence interval, 79.09 to 202.03). AFG treatment improved the survival of mice in a dose-dependent manner; however, a maximal response was not achieved with either isolate even in those treated with the highest AFG dose.

Aspergillus fumigatus may cause life-threatening infections in both immunocompetent and immunocompromised patients (1–3). Voriconazole (VCZ) is considered the first choice of therapy for invasive aspergillosis (IA) (4, 5). However, the rate of azole resistance is increasing in *A. fumigatus*, which significantly complicates the management of IA, as azole resistance is associated with therapeutic failure and a mortality rate of up to 88% (6–13). Primary invasive infections due to resistant isolates involving the lung (13, 14), bone (15) and brain (14, 16) have been reported, as have respiratory isolates in patients with allergic bronchopulmonary aspergillosis (7). Seventy-nine percent of isolates with the TR_{34}/L98H mutation are VCZ resistant (VCZ^r), and this mutation is the most prevalent resistance mechanism in clinical isolates (11). All patients with pulmonary aspergillosis due to TR_{34}/L98H mutant isolates who received VCZ monotherapy died by the 12th week of therapy (11). Therefore, it is important to explore alternative treatment regimens, as alternative treatments may improve the therapeutic outcome in patients with azole-resistant IA.

Anidulafungin (AFG) belongs to the echinocandins but has a unique site of action different from that of azoles and polyenes, as it targets cell wall synthesis, and has fungistatic activity against *Aspergillus* spp., in addition to an excellent safety profile (17–19). Little is known about the *in vivo* efficacy of the echinocandin AFG in IA.

Here we investigated the pharmacokinetic (PK)-pharmacodynamic (PD) properties of AFG in a nonneutropenic murine model of IA. For this purpose, we used two clinical isolates with different profiles of susceptibility to voriconazole: a VCZ-susceptible (VCZ^s) *A. fumigatus* isolate and a VCZ^r *A. fumigatus* isolate harboring a TR_{34}/L98H mutation in the *cyp51A* gene.

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MATERIALS AND METHODS

Fungal isolates. Two clinical *A. fumigatus* isolates obtained from patients with proven IA were used in the experiments: a VCZ^s isolate without mutations in the *cyp51A* gene (AZN 8196) and a VCZ^r isolate (V52-35) harboring the TR_{34}/L98H resistance mechanism. Strain identifications and the *cyp51A* gene substitutions were confirmed by sequence-based analysis as described previously (9). The isolates had been stored in 10% glycerol broth at −80°C and were revived by subculturing on Sabouraud dextrose agar (SDA) supplemented with 0.02% chloramphenicol for 5 to 7 days at 35 to 37°C. The *in vitro* antifungal susceptibility test was performed on the basis of EUCAST guidelines, using a broth microdilution format (20).

Infection model. A total of 170 outbred female CD-1 mice (age, 4 to 5 weeks; weight, 20 to 25 g; Charles River, the Netherlands) were randomized into groups of 17 mice for AFG monotherapy. Animals were infected using the procedure described before (21, 22). Before performing the experiment, the isolates were cultured once on SDA for 7 days at 35 to 37°C and subcultured twice on 15-cm Takashio slants for 5 days at 35 to 37°C. The conidia were harvested in 20 ml of sterile phosphate-buffered saline (PBS) plus 0.1% Tween 80 (Boom B.V. Meppel, the Netherlands). The conidial suspension was filtered through sterile gauze folded four

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TABLE 1 Origin, *in vitro* susceptibilities, and underlying azole resistance mechanisms of VCZ^s and VCZ^r *A. fumigatus* isolates

A. fumigatus isolate[a]	Cyp51A substitution	MIC (mg/liter)			Anidulafungin MEC (mg/liter)
		Amphotericin B	Posaconazole	Voriconazole	
AZN 8196	None	0.5	0.031	0.25[b]	0.031
V52-35	TR_{34}/L98H	0.5	0.5	4[c]	0.031

[a] The *A. fumigatus* isolates were from patients with proven invasive aspergillosis.
[b] Susceptible.
[c] Resistant.

times to remove any hyphae, and the number of conidia was counted in a hemocytometer. After the inoculum was adjusted to the required concentration, the conidial suspension was stored overnight at 4°C. The 90% lethal dose (LD_{90}) was separately determined for each isolate. Mice were infected via injection into the lateral tail vein of an inoculum corresponding to the LD_{90} of each isolate. The LD_{90}s of VCZ^s and VCZ^r (TR_{34}/L98H mutant) *A. fumigatus* isolates used in the current study were 2.4×10^7 and 2.5×10^7 conidia, respectively. Postinfection viability counts of the injected inocula were determined to ensure that the correct inoculum had been injected.

The animals were housed under standard conditions, with drink and feed supplied *ad libitum*. The animal studies were conducted in accordance with the recommendations of the European Community (Directive 86/609/EEC, 24 November 1986), and all animal procedures were approved by the Animal Welfare Committee of Radboud University (RU-DEC 2010-187). The infected mice were examined at least three times daily. These clinical inspections were carried out in order to ensure that there were no cases of desiccation, torticollis, staggering, high weight loss (a decrease of 15% within 48 h or 20% within 24 h), or body temperature drop to below 33°C. Mice demonstrating these signs of disease were humanely terminated. On day 15 postinfection, all remaining surviving mice were humanely euthanized under isoflurane anesthesia, and blood and internal organs were collected. The survival (in number of days postinfection) was recorded for each mouse in each group and was the outcome effect measure used to assess the therapeutic efficacy of AFG monotherapy (23).

Antifungal compound and treatment regimens. Treatment groups consisted of AFG (Pfizer, Capelle a/d IJssel, the Netherlands) monotherapy at 2.5, 5, 10, 20, and 40 mg/kg of body weight/day. Intraperitoneal therapy was begun at 24 h postinfection for 7 consecutive days and was given once daily with standard daily dosing, in addition to a single loading dose of AFG. The control group received single doses of saline.

Pharmacokinetic analysis of AFG in mice. A total of 144 outbred female CD-1 mice (age, 4 to 5 weeks; weight, 20 to 22 g; Charles River, the Netherlands) were used for separate PK experiments. On day 0, mice were infected with the wild-type *A. fumigatus* isolate through the lateral tail vein, and after 24 h, treatment was initiated, as described above, at dosages of 5, 10, 20, and 40 mg/kg AFG. At day 2 of treatment (day 3 after infection), blood samples were drawn through an orbital vein or by heart puncture and placed into lithium-heparin-containing tubes at 12 predefined time points: immediately before administration of drugs and subsequently at 0, 0.5, 1, 2, 4, 8, 12, 16, 20, 24, 48, and 72 h postdose. Blood samples were cooled and centrifuged for approximately 10 min at 1,000 × *g* within 30 min of collection. Plasma was aspirated, transferred into two 2-ml plastic tubes, and stored at −80°C.

Analytical assay of anidulafungin. Anidulafungin samples were measured by ultraperformance liquid chromatography (UPLC) with fluorescence detection. Samples were pretreated using a protein precipitation procedure (acetonitrile-methanol [50/50] and formic acid [0.1%]). A seven-point calibration curve with three quality control samples was used. All measurements were done in duplicate. The dynamic range of the assay was 0.008 to 8.4 mg/liter, and the accuracy range ($n = 15$), which was dependent on the concentration, was 94.2% to 103.5%. The intraday precision varied between 0.9% and 1.8%, and the interday precision was between 0.5% and 1.6%. Validation in mouse plasma was over the dynamic range of 0.008 mg/liter to 5.9 mg/liter. The intraday precision varied between 101.0% and 104.8%. Three freeze-thaw cycles did not impact the stability of anidulafungin. Geometric mean concentrations of AFG in plasma from three mice were separately calculated per time point. Maximum concentrations in plasma (C_{max}) were directly observed from the data. Pharmacokinetic parameters were derived using noncompartmental analysis with WinNonLin, version 5.2, software (Pharsight, Inc.). The area under the plasma concentration-time curve (AUC) from time zero to 24 h postinfusion ($AUC_{0\text{-}24}$) was determined by use of the log-linear trapezoidal rule. The elimination rate constant was determined by linear regression of the terminal points of the log-linear plasma concentration-time curve. The terminal half-life was defined as ln 2 divided by the elimination rate constant. Clearance (CL) was calculated as dose/$AUC_{0\text{-}24}$.

Statistical analysis. All data analyses were performed by using GraphPad Prism, version 5.0, software for Windows (GraphPad Software, San Diego, CA). A regression analysis was conducted to determine the linearity between dose and AUC. Mortality data were analyzed by the log rank test. The survival data were plotted against the dose/minimum effective concentration (MEC), and the Hill equation with a variable slope was fitted to the data both for each individual isolate and for pooled survival data. The goodness of fit was checked by use of the R^2 value and visual inspection. Statistical significance was defined as a P value of <0.05 (two-tailed). Dose/MEC and AUC_{0-24}/MEC ratios were calculated by dividing the dose (in milligrams per kilogram of body weight) or AUC by the MEC. Dose/MEC and AUC_{0-24}/MEC ratio data were $\log_{10}$ transformed to approximate a normal distribution prior to statistical analysis.

RESULTS

***In vitro* susceptibility.** The characteristics and *in vitro* susceptibility of the two selected *A. fumigatus* isolates are shown in Table 1. Both isolates grew well after 48 h of incubation at 35 to 37°C. VCZ showed reduced *in vitro* activity against the TR_{34}/L98H mutant isolate, with a VCZ MIC of 4 mg/liter for the TR_{34}/L98H mutant isolate compared to one of 0.25 mg/liter for the wild-type isolate. There was no difference in AFG activity, and both isolates had identical MECs.

Pharmacokinetics of AFG. A total of 144 mice (3 mice per time point, 12 time points, 4 different dosages) were analyzed. All 144 mice were alive at the time of sample collection. The observed plasma concentration-versus-time profiles of AFG are shown in Fig. 1. The corresponding pharmacokinetic parameters are tabulated in Table 2. The AUC normalized to a dose of 2.5 mg/kg resulted in ratios of 18.06, 18.6, 14.1, 16.3, and 20.1 for dosages of 2.5, 5, 10, 20, and 40 mg/kg, respectively. The AUC correlated significantly with the dose in a linear fashion over the entire dosing range ($R^2 = 0.86$).

Efficacy of AFG monotherapy. (i) Survival curves. The survival curves for all control groups receiving saline intraperitoneally showed a mortality of 90 or 100% and a median survival time of 3.5 to 4 days (Fig. 2). For both isolates, a dose-response rela-

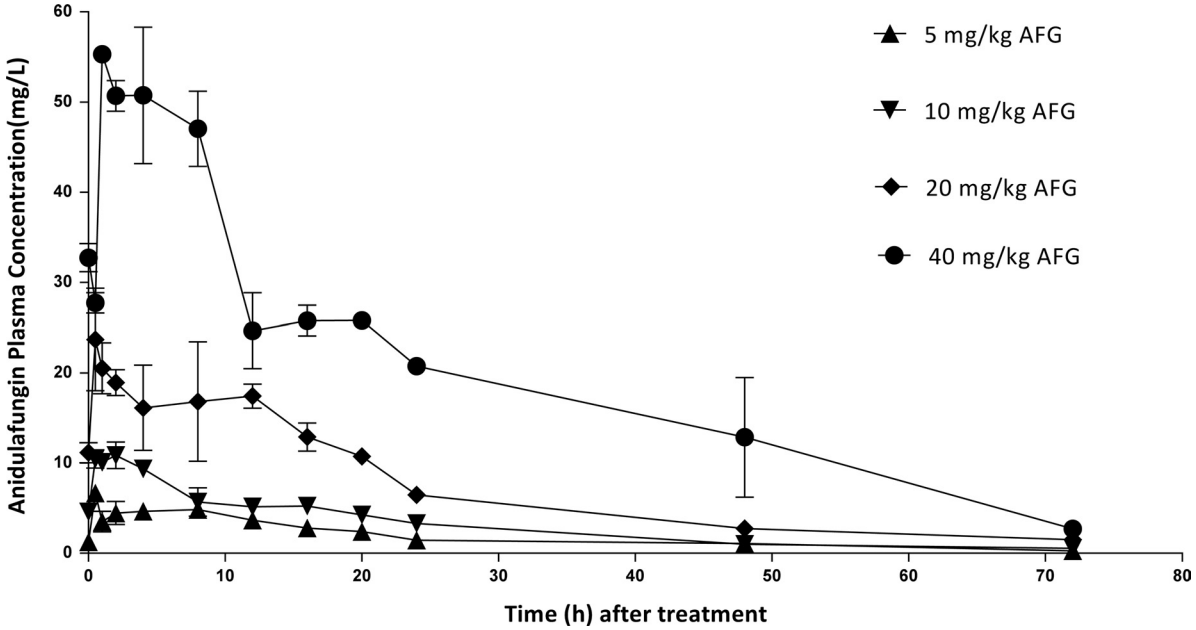

FIG 1 Plasma concentrations of anidulafungin following intraperitoneal administration of 5, 10, 20, and 40 mg/kg to immunocompetent infected mice. Each symbol corresponds to the geometric mean and standard error of the mean plasma levels for three mice.

tionship with increasing survival with increasing dose was observed.

The maximum dose of AFG resulted in 72.7% survival in mice infected with the VCZ^s isolate, whereas it resulted in 45.45% survival in mice infected with the VCZ^r isolate. Of note, the response was lower in those infected with the VCZ^r isolate than the VCZ^s isolate for each dose (Fig. 2).

(ii) Dose-response analysis. The dose-response curves for the dosing regimen and control groups of AFG monotherapy are shown in Fig. 3. AFG treatment improved the survival of the mice in a dose-dependent manner. The dose-response curve for mice infected with the VCZ^r isolate was shifted to the right compared to that for mice infected with the VCZ^s isolate, indicating that higher doses of AFG were required to achieve similar efficacy. In mice receiving AFG monotherapy, a maximal response could not be achieved with either isolate, even in those treated with the highest AFG dose.

(iii) Exposure-response analysis. The AUC for each dose, determined from PK experiments (Table 2), was used to calculate the AUC_{0-24}/MEC ratio for each isolate, as shown in Fig. 4. The exposure-response relationship had a sigmoidal shape. Increased AFG exposure was required to obtain maximum efficacy in mice infected with the VCZ^r isolate compared to those infected with the VCZ^s isolate. The Hill equation with a variable slope fitted the relationship between the 24-h AUC/MEC ratio and 14-day survival well ($r^2 = 0.87$), as statistically significant pharmacodynamic indices (PDIs) for single-agent regimens ($P < 0.05$). The 50% effective AUC for AFG was 126.5 (95% confidence interval, 79.09 to 202.03). We also determined the relationship between the *in vivo* efficacy and other PDIs, such as the cumulative percentage of a 24-h period that the drug concentration exceeded the MIC under steady-state PK conditions and the peak level (C_{max})/MEC (data not shown). However, AUC_{0-24}/MEC appeared to be the most important pharmacodynamic index correlating with efficacy.

DISCUSSION

Our animal model indicated that AFG monotherapy is moderately effective against isolates with a VCZ MIC within the susceptible range and in groups of mice infected with the resistant TR_{34}/L98H mutant isolate, which had a VCZ MIC of 4 mg/liter. Although increasing doses increased survival in a dose-dependent manner, a maximal response was not achieved with either isolate, even in those treated with the highest AFG dose (20 mg/kg of body

TABLE 2 Pharmacokinetic parameters of anidulafungin after intraperitoneal administration of various doses of AFG[a]

Dose (mg/kg)	AUC_{0-24} (mg · h/liter)	Dose-normalized AUC [(mg · h)/(liter · kg)]	T_{max} (h)	C_{max} (mg/liter)	C_{min} (mg/liter)	CL_{ss}/F [liter/(h · kg)]	$t_{1/2}$ (h)
2.5	46.5[b]	18.6[b]					
5	93	18.6	8	7.9	0.82	0.05	17.3448
10	141.4	14.1	2	10.7	3.3	0.07	17.3849
20	326.3	16.3	0.5	22.2	6.4	0.06	22.7847
40	802.7	20.1	4	49.5	20.7	0.05	15.3336

[a] AFG doses of 2.5 to 40 mg/kg were used. Intraperitoneal therapy was begun at 24 h postinfection with standard daily dosing of AFG, in addition to a single loading dose of AFG. T_{max}, time to C_{max}; C_{min}, minimum concentration in plasma; CL_{ss}/F, apparent steady-state clearance; $t_{1/2}$, half-life.
[b] Simulated analysis of pharmacokinetic assay with concentrations ranging from 5 to 40 mg/kg.

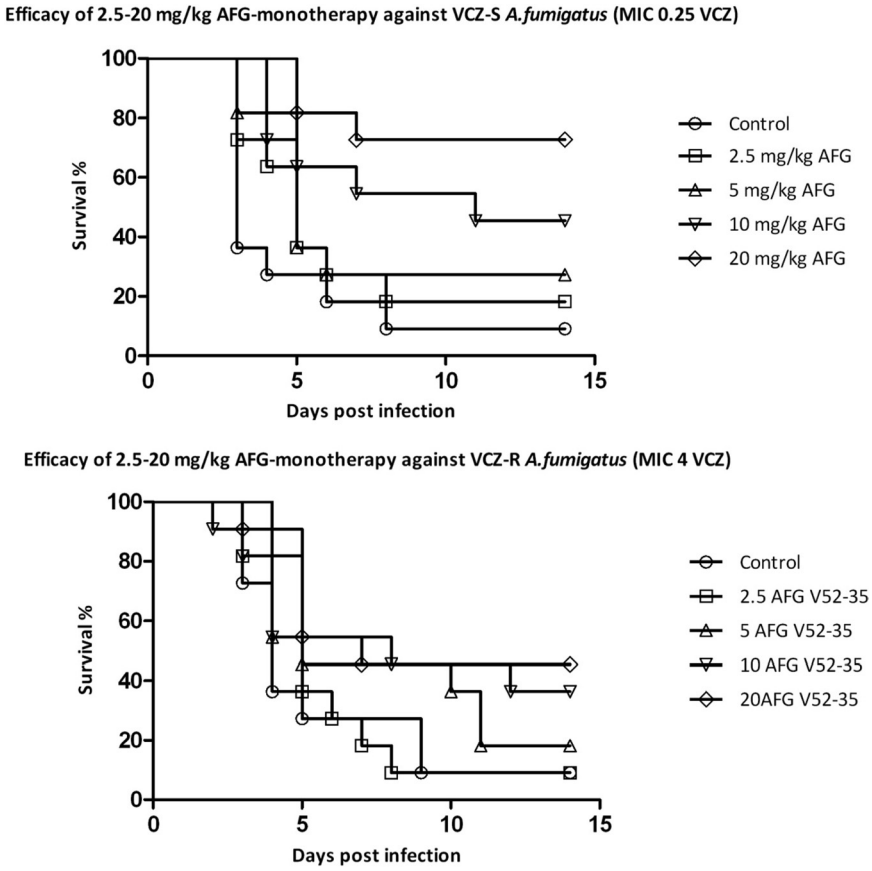

FIG 2 Efficacy of anidulafungin monotherapy against voriconazole-susceptible (MIC, 0.25 mg/liter) and voriconazole-resistant (MIC, 4 mg/liter) *A. fumigatus* isolates. Both isolates had the same AFG MEC (0.03 mg/liter). Control groups received saline. For all groups, $n = 11$.

weight). Apparently, this explains why AFG is not effective as single-drug therapy against *Aspergillus* infections, whereas VCZ is (24), confirming that AFG is a less potent drug for the treatment of IA (21, 22). A higher dose (40 mg/kg) of AFG was also studied for some groups in order to achieve higher efficacy; however, a dose-limiting toxicity was defined, and thus, we were not able to explore the effect of higher doses.

	AZN 8196	V 52-35
R square (weighted)	0.9927	0.9567

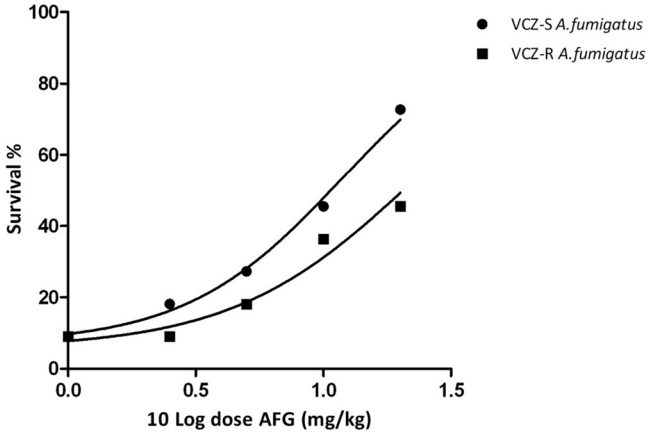

FIG 3 Anidulafungin dose-survival relationships for voriconazole-susceptible and voriconazole-resistant *A. fumigatus* isolates. The curves indicate fits with the Hill equation for each isolate.

Of note, AFG appeared to be slightly less effective against the VCZ^r isolate than the VCZ^s isolate, despite identical MECs, which raises a possible concern regarding the efficacy of anidulafungin monotherapy for azole-resistant IA. Although this difference could be due to differences in the virulence of the two isolates, we have no indications that this is the case, as we have used this isolate in our previous animal models, and the LD_{90} inocula were almost identical (2.4×10^7 versus 2.5×10^7 conidia). We also investigated the fitness of both isolates using a growth kinetic system (25) but found no differences in germination times or growth rates (results not shown). An alternative possibility might be that changes in ergosterol biosynthesis through mutations in the *cyp51A* gene might have indirect effects on fungal cell wall synthesis. These changes might not be reflected in *in vitro* susceptibility, as the MEC may not be sufficiently sensitive to detect subtle differences in echinocandin drug activity. Further research into this phenomenon is needed through, for instance, determination of the levels of the glucan synthase target enzyme in azole-resistant *A. fumigatus* isolates. A range of resistance mechanisms should be investigated, as the effect on the cell wall might differ depending on the underlying mutations.

The exposure-response relationship of AFG indicated that improvement of survival for both VCZ^s and VCZ^r isolates was de-

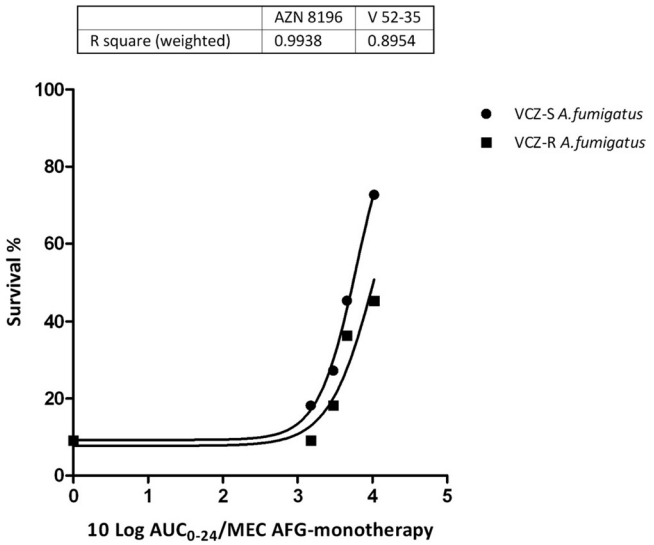

FIG 4 Percent of survival as a function of the anidulafungin AUC_{0-24}/MEC ratio for voriconazole-susceptible and voriconazole-resistant *A. fumigatus* isolates. Increased voriconazole and anidulafungin exposure was required to obtain maximum efficacy in mice infected by the voriconazole-resistant isolate. The curve is the model fit with the Hill equation for each datum.

pendent on the dose, and since the dose-AUC relationship was linear for the doses studies, this was also the case for the AUC_{0-24}/MEC ratio. The latter has relevance for predicting therapeutic efficacy (26).

In the present study, the AUC of total AFG was relatively high (326 mg · h/liter for the 20-mg/kg dose) and the MECs were quiet low (0.03 mg/liter), so that AFG treatment alone does not result in 100% survival. The major factor here is that AFG is highly protein bound, with protein binding estimated to be 99% (27) or possibly more. The AUC for the free, unbound fraction of AFG (*f*AUC)/MEC is therefore about 100 or even lower. The exact amount of free drug is not well-known, however, since protein binding at these high values is difficult to measure (27).

For echinocandins such as AFG, the 24-h *f*AUC/MIC ratio is considered the PK/PD index determining therapeutic efficacy, as indicated previously (26, 28). In humans, the AUC after a standard dose is slightly over 100 mg · h/liter (28, 29). Andes et al. found that a $fAUC_{0-24}$/MEC value of 100 was required to result in a static effect in a *Candida* infection model (27). This value is somewhat higher than the value found for other echinocandins, indicating that 99% protein binding may be an underestimation. van de Sande et al. reported only 18% survival of rats with IA after administration of AFG at human-equivalent doses. In this study, the steady-state $fAUC_{0-24}$ for AFG was calculated to be 120.3 μg · h/ml (29). Our results for AFG monotherapy at those values are in line with those reports. We also used higher doses, however, which resulted in increased survival of mice, although it did not reach 100%.

A possible limitation of the model used to explore the PK/PD relationships is that the effects were observed in nonneutropenic animals and the route of infection was dissemination rather than inhalation. However, IA in the nonneutropenic host is observed with increasing frequency, although other host factors might be impaired in such patients, in particular, those in an intensive care unit (3). The effects observed could therefore be an underestimation of the exposure required. On the other hand, studies with posaconazole and voriconazole in neutropenic (30) and nonneutropenic (21, 22) models have shown that the exposure-response relationships are of the same order of magnitude; in fact, slightly lower exposures were required in the neutropenic model.

With respect to the discussion presented above, the AUC_{0-24}/MEC appeared to be the most important pharmacodynamic index, which can be used to predict the outcome of AFG monotherapy. However, compared to the results of our previous study describing the pharmacodynamics of voriconazole monotherapy (24), the results of the present study indicate that AFG is less potent for the treatment of IA. Therefore, instead of using AFG monotherapy for IA, other treatment modalities including this agent in combination therapy can be useful approaches in the clinical setting to improve the therapeutic outcomes of patients with underlying IA. AFG belongs to the echinocandins, has a unique site of action, as it targets cell wall synthesis, and has fungistatic activity against *Aspergillus* spp. (18). The echinocandin AFG offers a particularly interesting option for combination antifungal therapy because of its mechanism of action, which is completely different from that of azoles and polyenes, and such combinations should be explored (17, 19). In addition, clinical studies have suggested that combinations of echinocandins with other antifungals are safe and may improve the response in patients with IA (17). However, preclinical studies and the results of a multicenter trial investigating such combinations will provide more data to judge this strategy.

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